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EFFECT OF NATURAL ANTIOXIDANTS ON THE LIPID STABILITY, PROTEIN INTEGRITY AND FLAVOUR OF PROCESSED MEAT PRODUCTS – SRP TRANSITION

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SRP Transition – Effect of Natural Antioxidants on the Lipid Stability, Protein Integrity and Flavour of Processed Meat Products

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Final Report

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EXECUTIVE SUMMARY

When the contract for the old SRP system terminated on March 31, 2003, SRP coordinators were asked by the University to prepare 5-year ADF project submissions. Mine was entitled "**Effect of Natural Antioxidants on the Lipid Stability, Protein Integrity and Flavour of Processed Meat Products.**" Later on we were informed that ADF had been looking for only submissions to cover the transition period. Hence, funding for the aforementioned project was awarded from ADF with the following three objectives:

1. Research must be limited to completion of bearberry extract for biological activities (e.g. antioxidants, anticancer, anti-inflammatory, *etc.*);
2. No commencement of research into new natural antioxidants;
3. A future research plan must focus on areas of pressing concern for Saskatchewan meat processors; and
4. Research must endeavour to establish collaboration with other leading Canadian researchers in meat chemistry.

Consequently, the title of this ADF project does not reflect the research which was asked for and completed. This report provides the scientific details pertaining to Objective 1. Regarding objective 4, I have collaborated with a few scientists in Canada and the United States on issues pertaining to meat chemistry. This has resulted in the publication of a number of book chapters and original scientific publications as presented in my CV. This is in addition to the meat technology/science studies carried out with Drs. Jag Dhanda, Phyllis Shand and Zeb Pietrasik at the University of Saskatchewan. Objective 3 was completed and submitted to Saskatchewan Agriculture & Food in a separate document.

Results of research into the health promoting potential of the bearberry-leaf extract have included the following: showing the interaction between meat protein and tannin constituents and proving that the resultant protein-tannin complexes still offer antioxidant activity; determining the chemical structures of the active tannin constituents that are providing antioxidant efficacy to cooked meat systems; identifying potential of the crude extract, low molecular-weight fraction and tannin fraction against cancer and chronic inflammatory disease using *in vitro* models.

TECHNICAL REPORT – PART I

Antioxidant Activity of Polyphenolics from a Bearberry-Leaf (*Arctostaphylos uva-ursi* L. Sprengel) Extract in Meat Systems

Executive Summary

Bearberry-leaf (*Arctostaphylos uva-ursi* L. Sprengel) extract possesses marked antioxidant activity in model and meat systems. A crude ethanolic extract of bearberry leaves was dechlorophyllized using a silicic acid column, and then fractionated by Sephadex LH-20 column chromatography using ethanol (95%, v/v) and acetone (50%, v/v) as the mobile phases. According to a mass balance, the ethanol fraction comprised 79.2% of the starting material, while the acetone fraction consisted of 9.7%. The content of total phenolics for the fractions and subfractions ranged from 2135 to 9110 Abs_{725} units/g extract. Even though the acetone fraction was only *ca.* 10% of the crude extract, its vanillin response was five times greater than that of the ethanol fraction. According to a mass balance of this fraction, *ca.* 50% of the polyphenolics remained in the aqueous phase when partitioned between water and ethyl acetate.

Results showed that the crude bearberry-leaf extract, its ethanol and acetone fractions inhibited TBARS formation in cooked meat systems after seven days of refrigerated storage by 97.0, 49.1 and 100%, respectively, when added at a 200-ppm concentration. The ethanol fraction showed a classical dose response. When incorporation to meat systems at levels of 200- and 500-ppm before thermal processing, TBARS development was inhibited by 49.7 and 93.9%, respectively, after seven days. This [ethanol] fraction was further subdivided into vanillin-positive constituents; the vanillin-positive fraction possessed weak antioxidant activity in meat model systems, and in some cases demonstrated a slight pro-oxidant effect. The acetone fraction also showed a classical dose response. When added to meat systems at levels of 25-, 50- and 100-ppm, TBARS formation was inhibited by 36.7, 91.4 and 100%, respectively, after seven days of refrigerated storage. It was interesting to note that a subfraction from the acetone product, which was soluble in ethyl acetate but did not have a positive reaction with vanillin, imparted strong antioxidant activity in meat systems. Therefore, vanillin-positive reaction constituents (*i.e.*, tannins) are not solely responsible for the antioxidant activity observed from the bearberry-leaf extract.

Background

Spices and herbs are not only used to flavor foods, but they also offer preservation against microbiological and oxidative degradation. Constituents in spices and herbs have antioxidant activity and can retard rancidity resulting from lipid oxidation and thereby help to preserve the wholesomeness of food products. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ) are examples of some common synthetic antioxidants used by food processors in their formulations. Yet, concerns have been raised over the safety of some of these food additives (*1*). Moreover, the concept of synthetic and chemical does not fit in with the consumers' growing interest toward using constituents that

are either natural or organic in food products. There are a number of natural antioxidants added to foods, some of which are depicted in Figure 1. Ascorbic acid and α-tocopherol (*i.e.*, vitamin C and E) are the best known water- and lipid-soluble antioxidants, respectively; they are added to food products as well as being found naturally in them. In addition, carotenoids, simple phenolic acids and phenolics from the flavonoid family, such as rutin (*i.e.*, a flavone), naringin (*i.e.*, a flavonone), kaempferol (*i.e.*, a flavonol), genistein (*i.e.*, an isoflavone) and (+)-catechin (*i.e.*, a flavanol) are commonly found in the foods we eat.

Researchers continue to search for novel sources of natural antioxidants from plants for use in food systems. Amarowicz *et al.* (*2*,*3*) screened selected plant species for their phenolic content, as it was realized that polyphenolics can function as strong antioxidants. Emphasis was placed, but not limited to, species thriving on the Canadian Prairies. From this research, it was determined that a crude ethanol extract from bearberry (*Arctostaphylos uva-ursi* L. Sprengel) leaves possessed marked antioxidant activity in both model and food systems. Bearberry is one of the most ubiquitous herbs on the Canadian Prairies. Its main constituents are arbutin (5-15%), variable amounts of methylarbuitn (up to 4%) and small quantities of the free aglycones. Other constituents include gallic acid, *p*-coumaric acid, syringic acid, galloylarbutin and up to 20% gallotannins and catechol types, as well as some flavonoids, notably glycosides of quercetin, kaempferol and myricetin.

The antioxidant activity of the crude bearberry-leaf extract was assessed by a number of chemical assays including a β-carotene-linoleic acid (linoleate) model system (*i.e.*, monitoring the coupled oxidation of β-carotene and linoleic acid), reducing power, scavenging effect on DPPH• free radical, liposome model system, scavenging capacity of hydroxyl free radicals (HO•) by use of electron paramagnetic resonance (EPR) spectroscopy, and ability to curb lipid oxidation in meat model systems. Some results from these tests are reported by Amarowicz *et al.* (*3*). Amarowicz and Pegg (*4*) fractionated the crude bearberry-leaf extract and found that the tannin constituents play a significant role toward the antioxidant activity observed in model and food systems.

Tannins are complex secondary metabolites of plants, which are widely distributed in foods and feeds. They are comprised of gallic acid esters or flavan-3-ol polymers and have molecular weights typically in the range of 500 to 3,000 Da (*5*), although the existence of larger tannins have been reported. Tannins can form soluble and insoluble complexes with proteins; the formation of these depends not only on the size, conformation and charge of the protein molecules, but also the molecular weight, length and flexibility of the tannins involved (*6*). The precipitation of a tannin-protein complex results from the formation of a sufficiently hydrophobic surface on the complex. At low protein concentrations, precipitation is due to the formation of a hydrophobic monolayer of polyphenols on the protein surface. At higher concentrations, however, a hydrophobic surface results from the combination of complexing of polyphenols on the protein surface and crosslinking of different protein molecules with polyphenols (*7*). Tannins bound to proteins have been shown to retain their antioxidant activity and may provide persistent antioxidant activity in the gastrointestinal tract when consumed (*8*).

The purpose of this part of the study was to further investigate the tannin constituents fractionated from the bearberry-leaf extract as well as the antioxidant activity they can afford to a meat model system.

Materials and Methods

Preparation of Bearberry-Leaf Extract: Dried bearberry leaves were ground in a coffee mill (Moulinex Corporation, Toronto, ON). Prepared material was transferred to dark-colored flasks, mixed with 95% (v/v) ethanol at a material-to-solvent ratio of 15:100 (m/v) and placed in a shaking Magni Whirl constant-temperature bath (Blue M Electric Company, Model MSG-1122A-1, Blue Island, IL) at 50°C for 30 min. Afterwards, the slurry was filtered through Whatman No. 1 filter paper and the residue was re-extracted twice more. Combined supernatants were evaporated to dryness under vacuum at < 40°C using a Büchi Rotavapor/Water bath (Models EL 131 and 461, respectively, Brinkmann Instruments [Canada] Ltd., Mississauga, ON). The crude preparation was then dechlorophyllized according to Pegg *et al.* (*9*) on a silicic acid column using hexanes and 95% (v/v) ethanol as mobile phases. Dried extracts were stored at 4°C in air until further analysed.

Fractionation of the Crude Bearberry-Leaf Extract: Approximately 4 g of the crude dechlorophyllized bearberry-leaf extract was suspended in 20 mL of 95% (v/v) ethanol and applied to a chromatographic column (4.5 × 18 cm) packed with Sephadex LH-20 and equilibrated with 95% (v/v) ethanol. The column was exhaustively washed with 95% (v/v) ethanol at a flow rate of 400 mL/h and then eluted with 50% (v/v) acetone at a flow rate of 300 mL/h. Eluates for each solvent system were combined and the organic solvent removed under vacuum at < 40°C using the Rotavapor. The "purified" tannin fraction from the acetone extract was lyophilized to remove residual water. One hundred milligrams of each extract was dissolved in 25-mL water and transferred to a 100-mL separatory funnel. Twenty-five milliliters of ethyl acetate were added, the contents shaken and phases left to separate for *ca.* 20 min. Three liquid phases were evident: a clear, upper ethyl acetate layer, a turbid middle layer, and a bottom water layer. The ethyl acetate layer was collected by pipette and labeled as either EtOH Fr. II or Acetone Fr. II, while the turbid middle layer was carefully removed from the separatory funnel and labeled as either EtOH Fr. III or Acetone Fr. III. Another 25-mL portion of ethyl acetate was added to the funnel and the extraction/collection procedure was repeated nine more times. Solvents from each fraction were removed using the Rotavapor.

Total Phenolics in the Bearberry-Leaf Extract and its Fractions: The total phenolics' content in the bearberry-leaf extract and its fractions was estimated by a colorimetric assay based on procedures described by Swain and Hillis (*10*) and Naczk and Shahidi (*11*). Briefly, a 0.5-mL aliquot of plant material dissolved in methanol was pipetted into a test tube containing 8 mL of distilled water. After mixing the contents, 0.5 mL of the Folin & Ciocalteu's phenol reagent and 1 mL of a saturated solution of sodium carbonate were added. The contents were vortexed for 15 s and then left to stand at room temperature for 30 min. Absorbance measurements were

recorded at 725 nm using a Milton Roy Spectronic Genesis 5 spectrophotometer (Fisher). Estimation of the phenolic compounds was carried out in triplicate; the results were averaged and expressed as Abs_{725} units/g extract or fraction.

Tannin Contents in the Bearberry-Leaf Extract and its Fractions: The content of condensed tannins in extracts was determined colorimetrically using the modified vanillin assay (*12*) and the proanthocyanidin assay (*13*). For the vanillin assay, either 5 mL of 0.5% (w/v) vanillin reagent prepared with 4% concentrated HCl in methanol (sample) or 5 mL of 4% concentrated HCl in methanol (blank) was added to 1 mL of methanolic solution of the bearberry-leaf extract or its fractions and then mixed well. After standing for 20 min in the dark at room temperature, absorbance measurements of the blank and samples were recorded at 500 nm. The absorbance of the blank was subtracted from the absorbance of the sample to account for potential interference from pre-existing chromophores in the sample. (+)-Catechin was used as the standard; from the curve constructed the results were expressed as the content of tannins in mg of (+)-catechin equivalents per g extract/fraction. For the proanthocyanidin assay, 1 mL of methanolic solution of the bearberry-leaf extract or its fractions was added to 10 mL of *n*-butanol-HCl reagent (*i.e.*, 0.7 g of $FeSO_4 \cdot 7H_2O$ was dissolved in 25 mL of concentrated HCl containing a small volume of *n*-butanol; the solution was then made up to 1 L with *n*-butanol) and mixed well. The mixture was heated in sealed ampules for 2 h in a boiling water bath and then allowed to cool to room temperature. The absorbance of the solution was measured at 550 nm against a reagent-only blank. Results were expressed as absorbance units per 1 g extract or fraction (Abs_{550}/g).

Tannin-Protein Complex Formation Determinations: The effect of tannins at various concentrations resulting in the formation of an insoluble tannin-protein complex was assayed by the protein precipitation method of Hagerman and Butler (*14*) with a pH modification as described by Naczk *et al.* (*15*). A series of methanolic solutions of the bearberry-leaf extract or its fractions were prepared at concentrations ranging from 0.5 to 3 mg/mL. From the series, 1 mL of each was taken and mixed with 2 mL of a standard bovine serum albumin (BSA, Fraction V, Sigma-Aldrich Canada, Ltd., Oakville, ON) solution (*i.e.*, 1 mg of protein/mL in 0.2 M acetate buffer, pH 4.0, and containing 0.17 M sodium chloride). After 15 min of standing at room temperature, the solution was centrifuged for 15 min at maximum speed using an IEC clinical centrifuge. The supernatant was discarded and the surface of the pellet and tube walls were carefully washed with the acetate buffer (pH 4.0), without disturbing the pellet. The pellet was then dissolved in 4 mL of a sodium dodecyl sulfate (SDS) – triethanolamine solution (*i.e.*, 1% SDS and 5% (v/v) triethanolamine in distilled water); one milliliter of ferric chloride reagent (*i.e.*, 0.01 M $FeCl_3 \cdot 6H_2O$ in 0.01 M HCl) was added to it and mixed. Fifteen minutes after addition of the ferric chloride reagent, the absorbance of the resultant solution was read at 510 nm against a reagent blank consisting of 4 mL of SDS– triethanolamine solution and 1 mL of ferric chloride reagent. Absorbance readings at 510 nm were plotted against the amount of extract used in the assay. The protein precipitating capacity of the tannins was expressed as the slope of the resultant linear "titration curve" and reported as Abs_{510} units/mg extract or Abs_{510} units/mg (+)-catechin equivalents.

The biological activity of tannins in the bearberry-leaf extract and its fractions was assayed by the dye-labeled protein assay of Asquith and Butler (*16*). The BSA employed was covalently labeled with Remazol brilliant blue R (*i.e.*, a blue dye) according to Rinderknecht *et al.* (*17*), and as modified by Asquith and Butler (*16*), to provide a substrate which could be detected spectrophotometrically. Briefly, 1 mL of methanolic solution of the bearberry-leaf extract or its fractions (0.1-2.0 mg/mL) was added to 4 mL of blue BSA solution containing 2 mg of protein/mL in 0.2 M phosphate buffer, pH 3.8, as modified by Naczk *et al.* (*18*). The solution was mixed vigorously at 1000 rpm for 5 min at room temperature using a shaker Type 50000 Maxi-Mix III (Barnstead/Thermolyne, Dubuque, IA). The protein-tannin complex so formed was separated by centrifugation for 15 min at 5000 rpm. The supernatant was discarded and the surface of the pellet and tube walls were washed with the phosphate buffer, without disturbing the pellet. The pellet was then dissolved in 3.5 mL of 1% (w/v) SDS solution containing 5% (v/v) triethanolamine and 20% (v/v) 2-propanol. The absorbance was measured at 590 nm against an appropriate blank. As there is a fixed quantity of protein in the assay, the percent dye-labeled BSA precipitated due to tannin-protein complex formation was plotted against the amount of crude extract or fraction added to the reaction mixture. The protein precipitating capacity of the tannins was then expressed as the slope of the resultant linear "titration curve" and reported as the percent dye-labeled protein preciptated/mg extract or fraction or per mg (+)-catechin equivalents.

TBARS Determination: To monitor the antioxidant efficacy of the bearberry-leaf extract and its fractions, the development of 2-thiobarbituric reactive species (TBARS) over time was monitored using a pork model system. TBARS from stored cooked pork systems were analyzed using a trichloroacetic acid/phosphoric acid extraction assay. Briefly, a 5.0-g sample of ground pork, which had been treated with GTE and then thermally processed, was weighed into a sample bag. Fifty milliliters of a 20% (w/v) solution of trichloroacetic acid containing 1.65% of 85% (w/w) phosphoric acid was added to the bag *via* a Brinkmann dispensette, and the contents were blended for 2 min. A 50-mL aliquot of ice cold distilled water was added to the slurry and blended for an additional minute. The mixture was filtered through a Whatman No. 1 filter paper into a 100-mL volumetric flask and made to mark with a 1:1 (v/v) TCA:water. Five milliliters of the filtrate were pipetted into a polypropylene tube followed by 5 mL of 0.02 M 2-thiobarbituric acid (TBA) reagent. The tube was capped and contents vortexed. A blank was prepared using 5 mL each of 1:1 (v/v) TCA:water and TBA reagent. All tubes were heated in a boiling water bath for 35 min. After color development, the tubes were removed from the water bath, cooled in ice water for *ca.* 5 min and then absorbance readings taken at 532 nm. A standard curve was prepared using dilutions from a 0.2 mM 1,1,3,3-tetramethoxypropane working standard as described by Pegg (*19*).

$$\%Inhibition = (1 - \frac{Sample\ absorbance_t - TBHQ\ absorbance_{t=1}}{Nonadditive\ absorbance_t - TBHQ\ absorbance_{t=1}}) \times 100$$

where, sample absorbance$_t$ is the absorbance reading for the TBA-TBARS complex at 532 nm of the test sample at day t; TBHQ absorbance$_{t=1}$ is the absorbance reading for the synthetic

antioxidant treated control at day 1; and non-additive absorbance$_t$ is the absorbance reading for the control sample containing no additives at day t.

Results and Discussion

Preparation of the crude extract from bearberry leaves, its dechlorophyllization and fractionation on a lipophilic Sephadex LH-20 column, followed by purification steps using ethyl acetate:water (1:1, v/v) are depicted in Figure 2. Ethanol fraction I (*i.e.*, EtOH Fr. I) comprises the phenolics which eluted from the column when 95% (v/v) was used as the mobile phase. Thin layer chromatography plates (*i.e.*, silica gel with fluorescent indicator and a mobile phase of chloroform:methanol:water, 65:35:10, v/v/v) with a ferric chloride spray and UV lamp detection were employed to indicate when no more relevant phenolics eluted from the column. About 1 L of ethanol had passed through the Sephadex LH-20 column, at which point the mobile phase was changed over to 50% (v/v) acetone. A similar approach as described above was employed when collecting Acetone Fr. I, except that vanillin spray was used instead of ferric chloride to indicate when all of the tannins had eluted from the column. According to a mass balance, EtOH Fr. I comprised 79.2% of the crude dechlorophyllzied bearberry-leaf extract and Acetone Fr. I consisted of 9.7%. EtOH Fr. I was fractionated further/purified using ethyl acetate:water (1:1, v/v): EtOH Fr. II represents the phenolics extracted into ethyl acetate while EtOH Fr. III is the product of the middle cloudy layer between the upper ethyl acetate and bottom water phases of the separatory funnel. An analogous fractionation of Acetone Fr. I was performed.

The content of total phenolics and condensed tannins in the bearberry-leaf extract and fractions so obtained are presented in Table 1. Owing to the fact that the dominant phenolics in each fraction differ based upon the chromatography employed to obtain them, the results of total phenolics are expressed as Abs_{725} units per gram extract or fraction instead of as an equivalent to a monomeric unit from some polyphenolic. For example, the crude bearberry-leaf extract contains a substantial amount of arbutin, while Acetone Fr. I possesses more tannins. Consequently, construction of a calibration curve using an arbutin standard would be more appropriate in the first situation while using a flavanol such as (+)-catechin would be preferable in the latter. The content of total phenolics ranged from a low of 2135 Abs_{725} units/g for EtOH Fr. II (*i.e.*, purified using ethyl acetate) to a high of 9110 Abs_{725} units/g for Acetone Fr. I. Use of 50% (v/v) acetone as a mobile phase assisted in fractionating and concentrating the tannin constituents from the bearberry-leaf extract. Previous research has shown that a crude extract from bearberry leaf contains a significant amount of polyphenolics, notably tannins (*4*).

The results of the vanillin and proanthocyanidin assays are quite interesting. The vanillin assay is widely used for quantitative measurement of condensed tannins, or its monomeric components. The assay appears to be highly specific for a narrow range of flavanols and dihydrochalcones which have a single bond at the 2,3 position and free meta-orientated hydroxyl groups on the B ring (*20*). A positive reaction is indicated by the appearance of a light pink to deep cherry red coloration. Vanillin-positive compounds were detected in the crude extract as

well as EtOH Fr. I and Acetone Fr. I. Vanillin-positive compounds from EtOH Fr. I and Acetone Fr. I were not extracted by ethyl acetate (*i.e.*, they were absent from EtOH Fr. II), but were concentrated in the cloudy ethyl acetate/water and water layers. A marked concentration effect was noted for EtOH Fr. III. A parallel story is evident from the results of the proanthocyanidin assay. The data from the proanthocyanidin assay are reported as Abs_{550} units/g extract or fraction as opposed to a standard equivalent. The proanthocyanidin assay is carried out in butanol/HCl and depends on the acid hydrolysis of the interflavan bonds of condensed tannins to produce anthocyanidins. The presence of 3-deoxyproanthocyanidins, flavan-3,4-diols and flavan-4-ols in extracts will also produce heat stable anthocyanidins (*21*); these will react with the ferric ions employed in the colorimetric assay and therefore overestimate the absorbance readings. The yield of the reaction depends on the HCl concentration, temperature and length of the reaction time, proportion of water in the reaction mixture, and presence of transition metal ions as well as the length of the proanthocyanidin chain (*13*).

Selection of an appropriate standard for quantification is difficult as plant tannins are mixtures of polymeric compounds that differ in their sensitivity towards reagents used in their determination. The longer the polymer chain, the more anthocyanidin pigment is formed (*22*); thus, less of the red condensation product from the vanillin reaction is produced due to an increased steric hinderance towards the vanillin reagent (*23*). Assays of purified tannins have shown that the use of catechin equivalents overestimates the true tannin content (*12*). For example, authors reported that a tannin extract twice as concentrated as another would have twice the ΔA_{500}, but would appear to have more than twice as much tannin based on the catechin standard curve. Nevertheless, the vanillin assay is generally standardized with catechin, due to its structural relationship to condensed tannins. Table 1 reports the contents of condensed tannins from the vanillin assay expressed as mg catechin equivalents/g extract and from the proanthocyanidin assay as Abs_{550} units/g extract or fraction. The content of condensed tannins in the crude bearberry-leaf extract and its fractions ranged from 101 to 847 mg catechin eq./g extract and from 130 to 607 Abs_{550} units/g extract or fraction for the vanillin and proanthocyanidin assays, respectively. These levels are substantially greater than those found in the hulls of canola (*13*).

Although a number of protocols are available to estimate the protein precipitating capacity of tannins, the protein precipitation method described by Hagerman and Butler (*14*) and the dye-labeled BSA assay of Asquith and Butler (*16*) were selected for quantification of the protein precipitating potential of tannins from the bearberry-leaf extract and its fractions. The protein precipitation method estimates the amount of precipitated protein-bound tannins (*i.e.*, quantity of the insoluble tannin–protein complex formed), whereas the dye-labeled BSA assay permits the direct measurement of the amount of protein precipitated by tannins present in an extract or fraction. In the first method a "titration curve," showing the amount of tannin-protein complex formed *vs* increasing quantities of extract or fraction added to a solution containing a fixed amount of protein, is plotted. Quantitative incorporation of the tannins into the precipitate is assured by using at least twice as much protein as tannin, by weight (*14*). Due to the linear nature of the relationship, the slope is calculated. A larger slope denotes that the tannin constituents in the extract or fraction had a greater affinity to bind with the model protein (*i.e.*,

BSA), resulting in the formation of an insoluble tannin–protein complex. Results of the protein precipitating capacity of various fractions of bearberry-leaf phenolics are presented in Table 2 and indicated that bearberry polyphenolics are very effective protein precipitants. Acetone Fr. I was almost three times more effective than the crude extract at precipitating protein resulting in the formation of tannin–protein complexes. Knowing that Acetone Fr. I and EtOH Fr. I represent 9.7 and 79.2% of the crude extract, respectively, a calculated slope of 1.4 instead of 1.8 would be expected for the crude extract. The degree of polymerization of the polyphenolics and the presence of low molecular weight phenolics in crude preparations probably affect the extent of precipitation, thereby resulting in slight discrepancies from predicted and actual slope values. Nonetheless, the data generated from the protein precipitation method is in line with the results of the mass balance. Even though EtOH Fr. III was richer in condensed tannins than Acetone Fr. I, as evident by the vanillin and proanthocyanidin assays (see Table 1), it was not as effective as Acetone Fr. I at precipitating BSA and forming the tannin–protein complex. This suggests that other constituents besides condensed tannins – presumably hydrolyzable tannins – are present in Acetone Fr. I and facilitate protein precipitation. Hagerman and Butler (*14*) noted that their protocol was rapid, reproducible and could be used with either condensed or hydrolyzable tannins. When comparing the slope from the titration curve of Acetone Fr. I (*i.e.*, 5.10) with those of acetone fractions for beach pea, canola hulls, evening primrose and faba bean (*i.e.*, in the range of 0.25 to 1.13, from *6*), it can be seen that the fraction from the bearberry-leaf extract was 4.5 times stronger at precipitating BSA than the extract from canola hulls (*i.e.*, 1.13). Caution must be exercised, however, when comparing data in the literature. Besides modifications in methodology, a marked difference exists when results are expressed in Abs_{510} units/mg extract or fraction compared to when they are expressed as Abs_{510} units/mg catechin equivalents.

A similar story is seen from the results of the dye-labeled BSA assay. The numerical values of the slopes from the linear plots of % blue BSA precipitated *vs* increasing quantities of extract or fraction added to a solution containing a fixed amount of the dye-labeled protein ranged from 12.2 to 98.4. Introduction of the hydrophobic dye reduces the solubility of the BSA and makes it precipitate more readily than unlabeled BSA (*16*). Consequently, formation of a precipitate should not be interpreted as indicative of the presence of tannin unless corroborated by the protein precipitation assay. As with the data of the protein precipitation method, Acetone Fr. I was strongest at precipitating the bule BSA. The quantity of tannin present in an extract or fraction will affect its affinity for BSA resulting in precipitation, but so will the degree of polymerization. According to Porter and Woodruffe (*24*), the ability of condensed tannins to precipitate proteins depends on their molecular weight. It is particularly interesting to note that EtOH Fr. II, which was devoid of condensed tannins, had no affinity to complex with and precipitate the dye-labeled BSA. Furthermore, hydrolyzable tannins which were present in Acetone Fr. I (results not shown) are most likely absent from EtOH Fr. II. Clearly, further research is needed to characterize the tannin constituents from the bearberry-leaf extract.

Results of the antioxidant activity of the dechlorophyllized bearberry-leaf extract and its fractions in cooked pork systems after 7 days of refrigerated storage are summarized in Figure 3.

The active constituents present in the crude extract and its fractions showed excellent thermal stability during the cooking of the meat systems. This is significant, as a good number of natural antioxidants lose their capacity to quench free radicals or to chelate metal ions when subjected to food processing operations such as cooking. TBARS values of cooked pork model systems treated with a synthetic antioxidant (*i.e.*, TBHQ) as well as the bearberry-leaf extract and its fractions, at various concentrations, were compared. TBARS values of pork systems treated with the bearberry-leaf preparations were much lower than those of the control with no additive, thereby indicating protection to the meat lipids from constituents of the extract against autoxidation. Such data is better presented by reporting the % inhibition of lipid oxidation after a specified storage period in relation to two control systems: one without any additive where by autoxidation runs its course, and the second with the synthetic antioxidant at a suitable level to completely curb oxidation. Although an antioxidative efficacy was noticeable at 100 and 200-ppm levels, the crude bearberry-leaf extract at 200-ppm addition inhibited TBARS formation by 97% after 7 days of storage at 4°C storage. When the addition level of the extract was increased to 500 ppm, there was 100% inhibition of lipid oxidation over the same period.

The ethanol and acetone fractions recovered from the Sephadex LH-20 column gave some interesting data. EtOH Fr. I most likely contained simple phenolic acids, arbutin, methyl arbutin, gallic acid as well as condensed tannins, as indicated by the vanillin assay. Its antioxidant activity was weaker than that of the crude extract. Higher addition levels ~500 ppm were necessary to achieve the protection against autoxidation afforded by the crude bearberry-leaf extract at a 200-ppm concentration. On the other hand, Acetone Fr. I possessed a much stronger antioxidant activity than its precursor. As shown from the protein precipitating capacity data, this fraction was an effective protein precipitant and rich in hydrolyzable and condensed tannins. At a 50-ppm addition level, 91.4% protection in the cooked pork system was achieved after 7 days of refrigerated storage; when addition levels of 100 and 200 ppm were employed, complete protection against lipid oxidation, as assessed by the TBARS test, was achieved. Such results demonstrate the concentration dependence of additive inclusion in relation to observed antioxidant activity.

The further fractionation/purification of EtOH Fr. I and Acetone Fr. I produced some interesting results in terms of observed antioxidant activity. Both EtOH Fr. II and III imparted weak antioxidant activity to the pork systems when employed at 200 and 500-ppm levels. In fact, when lower levels than these were incorporated into the meat matrix, a slight pro-oxidant activity was observed. What is most interesting, however, is that EtOH Fr. III possessed a positive reaction with the vanillin test, thereby indicating the presence of flavan-3-ol end groups of condensed tannins. One would anticipate that such structural units should impart antioxidant activity, as observed for Acetone Fr. I which is rich in condensed tannins. This, however, was not the case. Concerning the data for Acetone Fr. II and III: both possessed strong antioxidant activity when applied to meat systems, but Fr. II gave a negative response with the vanillin reaction, again indicating the absence of condensed tannins. Hence, there are other constituents isolated in the acetone fraction – presumably the hydolyzable tannins – which imparted protection to the cooked pork systems against lipid oxidation. Characterizing the tannins

responsible for the observed antioxidant activity in food systems, such as the meat system described in this study, are described in Part II of this report.

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Personnel Involved

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Figure Caption List

Figure 1. Some typical natural antioxidants present in and added to food products.

Figure 2. Flow diagram for the preparation of the crude bearberry-leaf extract and its fractions.

Figure 3. Block diagram reporting the % inhibition of lipid oxidation of cooked pork systems treated with the crude bearberry-leaf extract and its fractions after 7 days of refrigerated storage. AA – antioxidant activity; ProA – pro-oxidant activity; - Vanillin rxn – negative reaction with the vanillin reagent; + Vanillin rxn – positive reaction with the vanillin reagent.

Table 1. Bearberry Fractions

Bearberry Fractions	*Total Phenolics*[a]	*Condensed Tannins*[b]	
		Vanillin	*Proanthocyanidin*
Crude Extract	4700±47	232±2	130±1
EtOH Fr. I	3360±161	124±4	160±4
EtOH Fr. II	2135±57	ND	ND
EtOH Fr. III	3480±61	847±46	607±37
Acetone Fr. I	9110±129	619±9	392±15
Acetone Fr. II	8380±132	ND	ND
Acetone Fr. III	3110±182	101±7	trace

NOTE: Results are mean values of at least three determinations ± standard deviation.
[a]expressed as Abs_{725} units/g extract or fraction.
[b]Vanillin assay is expressed as mg (+)-catechin equivalents/g extract or fraction; Proanthocyanidin assay is expressed as Abs_{550} units/g extract or fraction; ND – not detected.

Table 2. Protein precipitating capacity of various fractions of bearberry phenolics expressed as a slope value from titration curves[a]

Bearberry Fractions	*Protein Precipitation Assay*	*Dye-labeled BSA Assay*
	Abs/mg extract or fraction	*%PP/mg extract or fr.*
Crude Extract	1.82±0.03	16.7±0.5
EtOH Fr. I	1.12±0.02	12.2±0.6
EtOH Fr. II	ND	ND
EtOH Fr. III	3.55±0.06	57.3±4.9
Acetone Fr. I	5.10±0.15	98.4±4.9
Acetone Fr. II	2.20±0.47	54.9±1.8
Acetone Fr. III	2.45±0.10	31.3±3.2

[a]titration curves are lines that reflect the amount of proteins or tannins precipitated as a protein–tannin complex with increasing quantities of extract or fraction added to a solution containing a known amount of protein. Abs is absorbance at 510 nm; %PP is % dye-labeled BSA precipitated; ND – not detected.

TECHNICAL REPORT – PART II

Structure Elucidation of the Dominant Antioxidant Constituents Found in the Tannin Fraction of the Bearberry-Leaf (*Arctostaphylos uva-ursi* L. Sprengel) Extract

Executive Summary

The crude bearberry-leaf (*Arctostaphylos uva-ursi* L. Sprengel) extract was fractionated on lipophilic Sephadex LH-20 into a tannin and non-tannin fraction according to the procedures established in Part I of this report. To help isolate the most active antioxidant constituents, the tannin fraction was subjected to centrifugal partition chromatography (CPC) at the POS Pilot Plant under the direction of Dr. Udaya Wanasundara, Dr. Amarowicz and myself. Two fractions were obtained but some clean up was needed, so they were then subjected to semi-preparative HPLC. Three samples were obtained whose purity was confirmed by analytical HPLC; these were labeled HTAN1, HTAN2 and HTAN3. Electrospray mass spectrometry studies suggested that the three compounds were hydrolysable gallotannins, differing by only the number of gallic acid residues attached to the central glucose molecule. 1D and 2D NMR studies were performed at the Saskatchewan Structural Sciences Centre to obtain the necessary data for the Structure Elucidator, a computer NMR program from Advanced Chemistry Development (ACD), Inc. (ACD) to elucidate the structure of the unknown compounds. The ACD/Structure Elucidator confirmed that the three compounds possessing the strongest antioxidant activity of the bearberry-leaf extract were indeed hydrolysable gallotannins containing 3, 4 and 5 gallic acid moieties. All gallic acid moieties were etherifed to the hydroxyl groups of the central glucose molecule. When the bearberry-leaf extract is used as a natural antioxidant in food systems, the chemical structure of the dominant compounds imparting antioxidant activity can be given. Knowledge of the active compounds is important in the next step toward achieving regulatory status of this extract as a food additive.

Background

The ACD/Structure Elucidator is a software program that analyzes 1D and 2D NMR spectra, infrared (IR) spectra, mass spectra (MS), and elemental analysis (EA) to elucidate a chemical structure. It combines ACD/Labs spectral prediction and analysis engines with pseudo-artificial intelligence algorithms to achieve a "pseudo reverse 2D NMR predictor". That is, it takes 2D NMR data along with information about the molecular formula and generates a set of possible structures that describe the input data. It then goes one step further to help the scientist evaluate the structures by calculating the deviation between the spectral properties predicted for each generated structure and the experimental data.

At the core of ACD/Structure Elucidator is a set of three software engines, or generators, that deduce structural information from spectroscopic data by different means. This report illustrates the use of only the Correlation Spectroscopy-Based (CSB) generator, which requires 2D NMR data and molecular formula (MF). Spectral overlap makes structure elucidation of compounds containing more than 15-20 skeletal atoms difficult without 2D NMR data, therefore, the CSB

generator is preferred in such cases. The Classical generator requires only 1D ^{13}C NMR data and a molecular formula. Normally, the Classical generator is used for unknown molecules having less than 15-20 skeletal atoms. The Standard generator is used in situations where no 2D NMR information and no MF/MW information are available. The Classical and Standard generators can also use the additional information mentioned for the CSB generator, with the exception of 2D NMR data.

Presented below was the experimental approach taken in attempting to elucidate the structure of HTAN2. Numerous screenshots have been included to provide the reader with some insight into the program interface.

Materials and Methods

Computational Equipment: This study was conducted using version 9.03 of the ACD/Structure Elucidator program on a Pentium III 1 GHz system equipped with 512 MB RAM and the Microsoft Windows 2000 operating system. The screenshots were prepared on a computer using Microsoft Windows XP Professional (SP2).

Experimental Data: The following data was received for the unknown: MF = $C_{34}H_{28}O_{22}$, MW = 788 g/mol. Information pertaining to the presence of substituted glucose and benzene included ^{13}C NMR spectrum (processed and raw data); ^{1}H NMR spectrum (processed and raw data); $^{1}H/^{13}C$ HSQC spectrum (processed and raw data); $^{1}H/^{13}C$ HMBC spectrum (processed and raw data); and $^{1}H/^{1}H$ COSY spectrum (processed and raw data).

Results

After processing and peak picking in ACD/NMR Processor, the data was transferred to ACD/Structure Elucidator using a menu-command within the Processor window. Figures 1a and 1b display a set of tabular NMR data and its auto-assigned labels.

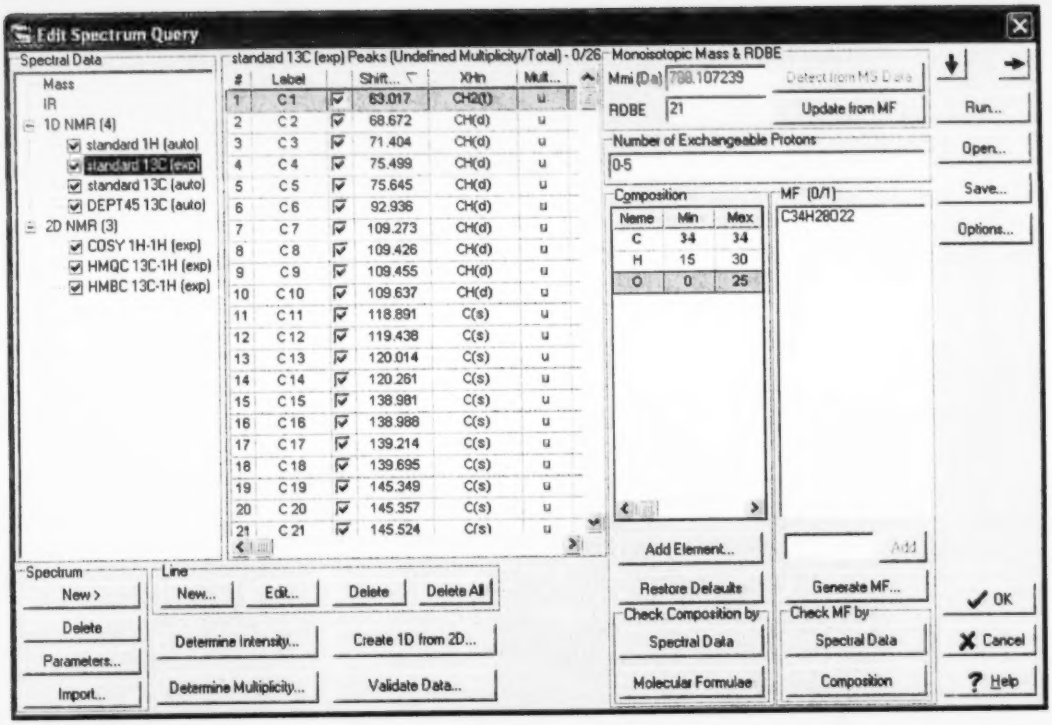


Figure 1a. The ACD/Structure Elucidator NMR Data Entry Interface listing all data, *e.g.* NMR, MW, MF, *etc.* The NMR data can be edited in this interface.

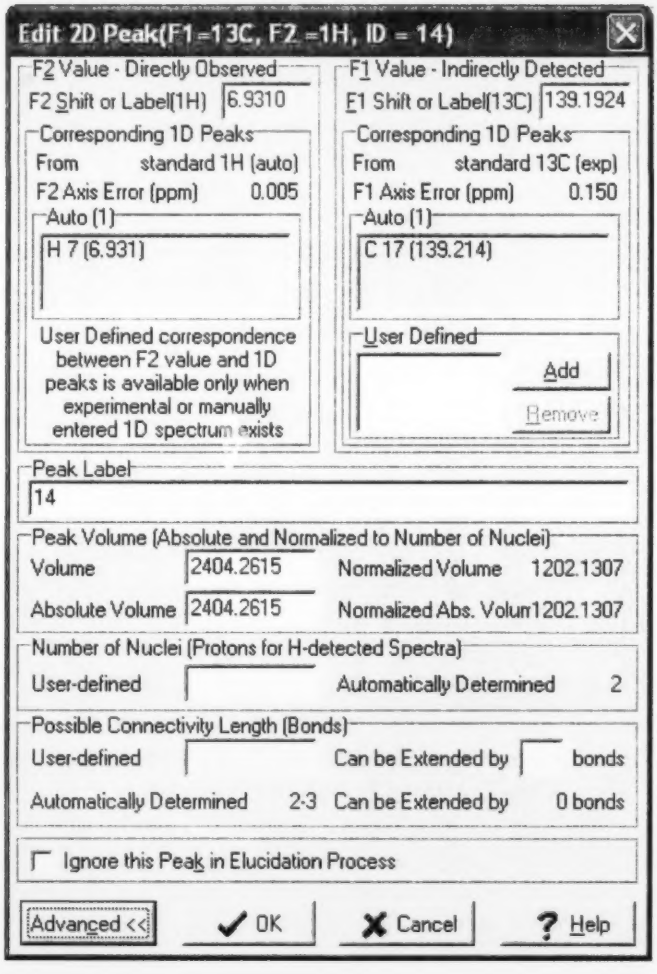


Figure 1b. The 2D NMR Peak Editing Interface of ACD/Structure Elucidator.

Determining the Molecular Formula: For this elucidation, the submitted MF was used from the beginning. However, the software does possess a MF generator. For this elucidation, the MW information and the NMR data can be incorporated into the software's MF generator.

As with any manual elucidation, having more than one MF can increase the time spent on elucidating an unknown. Yet, with the ACD/Structure Elucidator any modifications made on a single dataset can be applied to all MFs and their corresponding Molecular Connectivity Diagram (MCD) (MCD – See Figure 4).

Figure 1c shows the MF Generator for the molecular weight (MW) information of 788 +/- 0.5 Da. Using chemical shift information, the MF can be dwindled down to a single possibility.

1 MF was generated based on the following restrictions:

1. 34 Carbons
2. 15-30 Hydrogens
3. 0-25 Oxygens

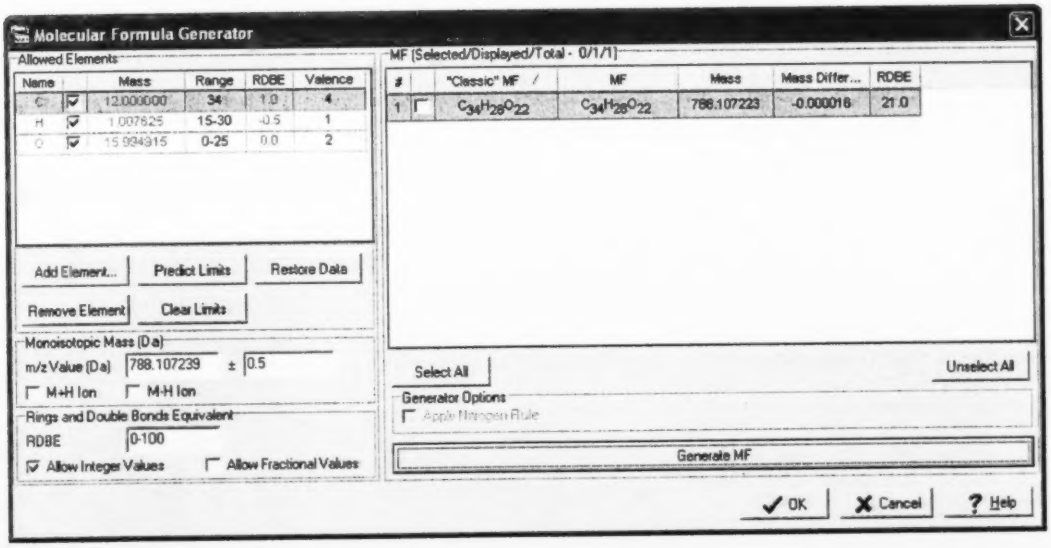


Figure 1c. The MF Generator Interface of ACD/Structure Elucidator.

2D NMR Interpretation Parameters: Cross-peak intensity in a 2D NMR spectrum is usually proportional to the active coupling constant, which depends (in part) on the number of intervening bonds between the coupled nuclei. In addition, the intensity information can often be roughly correlated with the distances represented by a cross-peak. This is one key to the way ACD/Structure Elucidator extracts connectivity information from 2D NMR data.

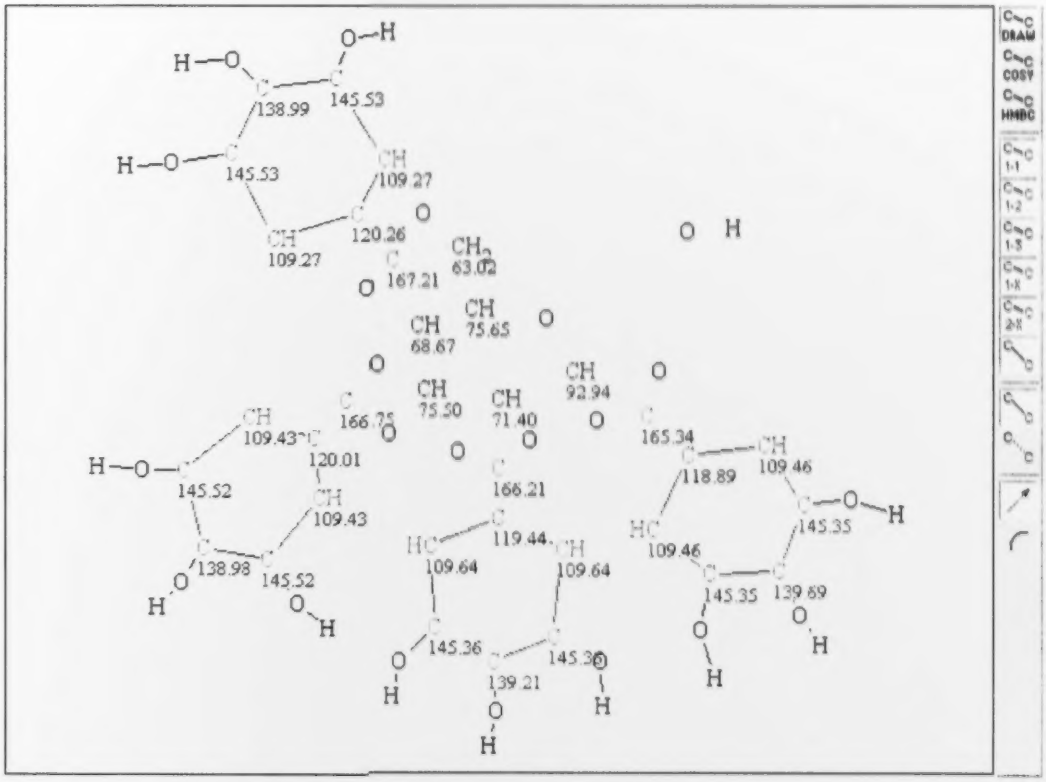
Because of the ambiguity inherent to the relationship between topological bond distances and cross-peak intensities, ACD/Structure Elucidator provides the ability to specify a range of bond lengths that a given correlation may imply. The default ranges (2-3 for COSY, 1 for HMQC, 2-3 for HMBC) provide the best compromise between elucidation time and the structural generality for most compounds. However, the default ranges can be easily overridden by entering values for the Minimum and Maximum Connectivity Length parameters shown in dialog box shown in Figure 1b.

Bond Terminology: For the purposes of structure elucidation, topological bond "distances" are defined as the number of covalent bonds along the coupling path between the *specific* atoms being referenced. Thus, when discussing a COSY spectrum, geminal protons are separated by 2 bonds, vicinal protons are separated by 3 bonds, *etc.* In the context of a molecular connectivity diagram (MCD), only skeletal atoms are considered, and so the distance between vicinal protons would be referred to as a 1 bond. The ACD/Structure Elucidator derives these bond "distances" from the presence and intensity of the cross-peaks in 2D NMR data. These distances are then used to provide constraints on the myriad of structures available from the molecular formula and standard element valences.

The HMQC correlations chosen were assumed to indicate connectivity across a single bond, while the HBMC data used was assumed to indicate a connectivity path including one or two skeletal bonds. From the NMR data, a Molecular Connectivity Diagram (MCD) was created.

In Figure 2, the bottom MCD displays numerous ambiguous bonds due to the crowding of 13C chemical shifts. To compensate for this, and thus simplify the problem, the following were performed manually:

1. Twelve OH bonds were created.
2. Four benzene rings were drawn based on user information.



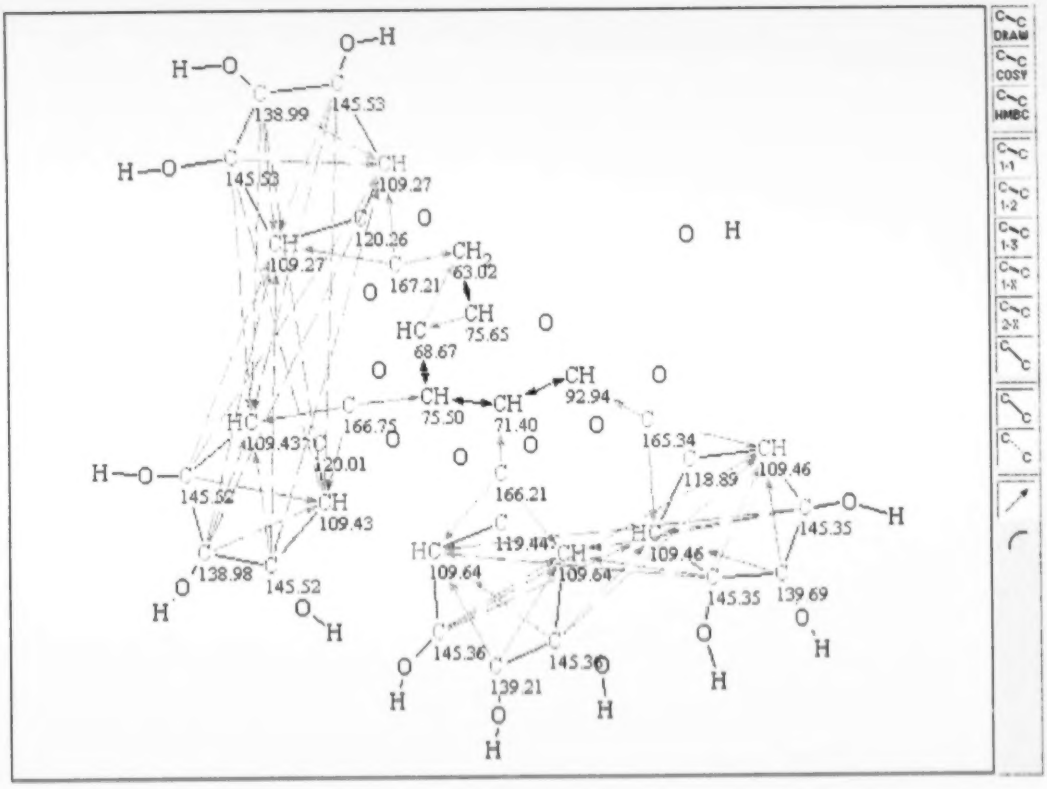


Figure 2. One MCD (Molecular Connectivity Diagram) was used for the elucidation process. The top MCD illustrates the manually created bonds while the bottom MCD illustrates all NMR connectivity information. Blue, and green lines represent 1-bond and 1-2 bond separation, respectively. Bonds drawn manually are shown in black. Dotted lines represent ambiguous connectivities. The experimental chemical shifts are indicated in blue for the carbons. The colouration of the carbons represents the hybridization, namely, blue for sp3, purple for sp2, and black for undefined. Note that the coordinates of the atoms have been positioned manually for better presentation.

Filter Procedures: From the generated MCD, 3 structures were generated utilizing the following specifications:

1. The maximum bond multiplicity was set to 2, *i.e.*, no triple bonds were allowed, and
2. Bonds between heteroatoms were not allowed.

The three ranked structures are shown in Figure 3a.

Once generated, the structures were ranked according to the deviation between the predicted NMR spectrum and the experimental spectrum. The calculated average deviation is a

comparison of experimental ^{1}H and ^{13}C NMR data with corresponding predictions. Compounds with a carbon match factor (d(^{13}C)) of less than 6 are the most likely candidates. A poor value is a result of either the incorrect structure has been elucidated, and/or the internal database of the predictors poorly represents the unknown compound. The second option can be improved by training the database, thus creating a user database, where similar compounds, derivatives and synthetic precursors can be added. For future structure determinations of related compounds, the addition of this compound to a system-training database would be a valuable asset in reducing generation times.

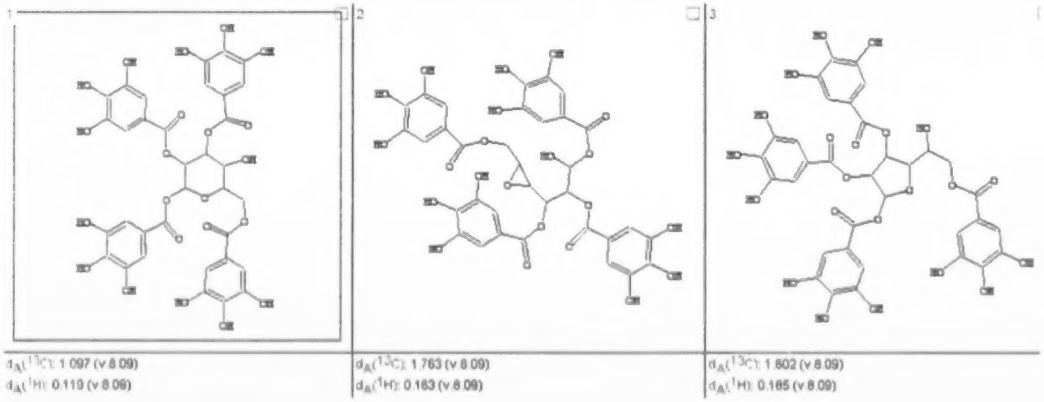


Figure 3a. The three structures ranked by CNMR deviation. The top structure generated, 1,2,3,6-tetrakis-*O*-(3,4,5-trihydroxybenzoyl)hexopyranose, has the lowest deviations, 1.097 ppm for carbon and 0.119 ppm for proton. Lower values indicate a better match between the experimental and calculated chemical shifts.

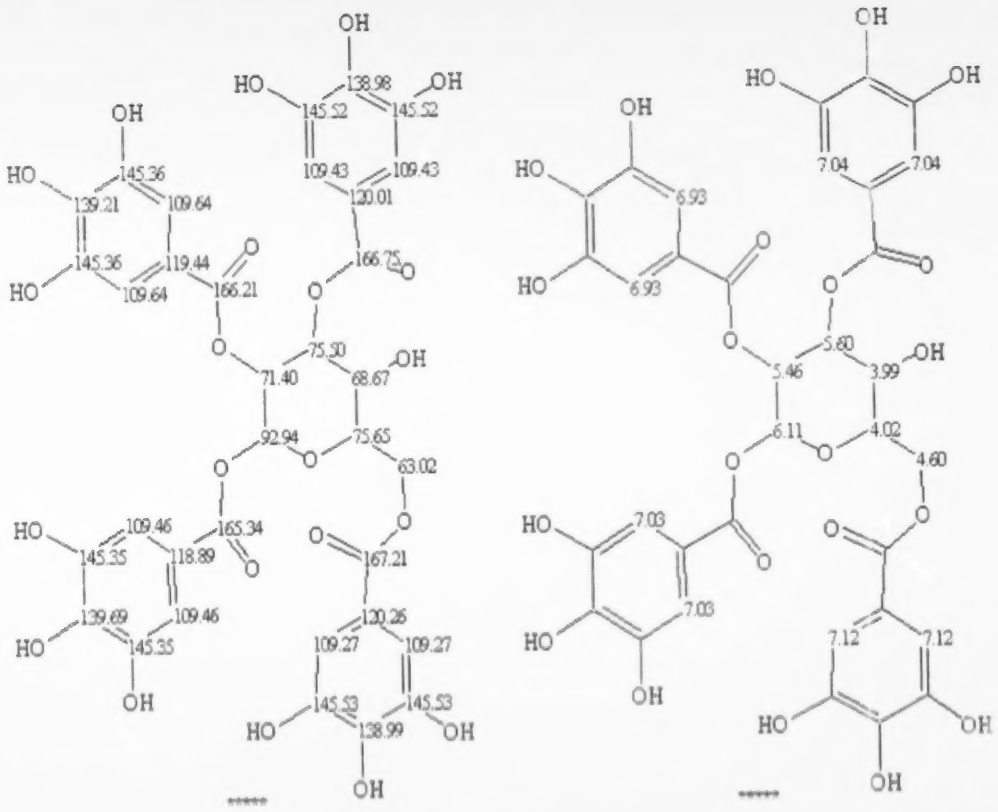


Figure 3b. The experimental ^{13}C and ^{1}H chemical shifts for 1,2,3,6-tetrakis-*O*-(3,4,5-trihydroxybenzoyl)hexopyranose.

As an additional filter, the generated structures can be limited by ring size by specifying a range (see Figure 4). Prior knowledge of the synthetic precursors or similar compounds can be used to define Obligatory and Forbidden ring sizes.

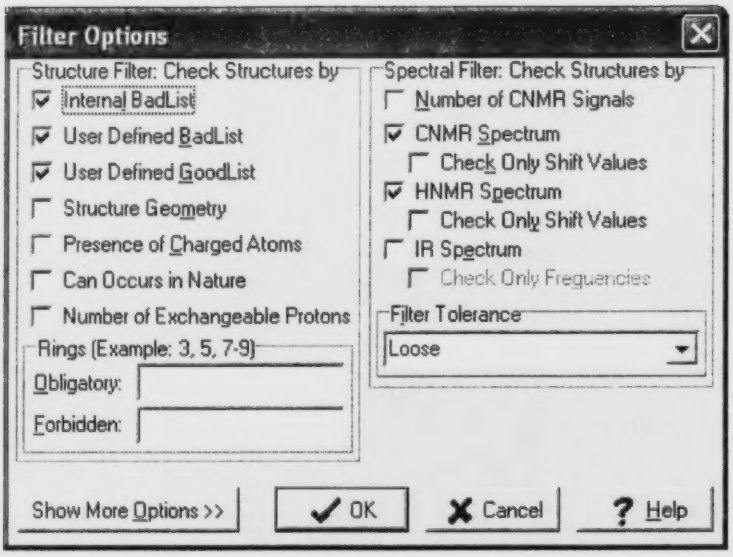


Figure 4. The Filter Options dialog box shows the criteria used to filter generated structures. Inclusion of prior information about the sample, such as ring size, will significantly narrow the number of possible molecules, and thus reduce elucidation time.

Conclusion

The results obtained using ACD/Structure Elucidator to solve HTAN2 are reported above. The unknown is believed to be 1,2,3,6-tetrakis-*O*-(3,4,5-trihydroxybenzoyl)hexopyranose. The carbon deviation factor has been calculated to be 1.097 ppm. For HTAN1 and HTAN3 there are one less and one additional gallic acid groups, respectively, etherified to the hydroxyl groups of the central glucose molecule. Complete characterization of the dominant antioxidant constituents of the bearberry-leaf extract has been achieved.

This challenge also demonstrates another valuable capability of the program is its thorough sampling of the solution space enables it to suggest possibilities that even a highly experienced human analyst may overlook.

Appendix : Spectral Peak Tables as used in the Elucidation

1H NMR

#	Label	Shift (ppm)	Multiplicity	Atoms	Height	Volume
1	H 10	7.123	u	2	2	2

2	H 9	7.04	u	2	2	2
3	H 8	7.027	u	2	2	2
4	H 7	6.931	u	2	2	2
5	H 6	6.107	u	1	1	1
6	H 5	5.601	u	1	1	1
7	H 4	5.461	u	1	1	1
8	H 3	4.595	u	1	1	1
9	H 2	4.025	u	1	1	1
10	H 1	3.989	u	1	1	1

13C NMR

#	Label	Shift (ppm)	XHn	Atoms	Height	Volume
1	C 1	63.017	CH2(t)	1	0.419	0.419
2	C 2	68.672	CH(d)	1	0.513	0.513
3	C 3	71.404	CH(d)	1	0.351	0.287
4	C 4	75.499	CH(d)	1	0.355	0.35
5	C 5	75.645	CH(d)	1	0.329	0.337
6	C 6	92.936	CH(d)	1	0.327	0.257
7	C 7	109.273	CH(d)	2	0.908	0.908
8	C 8	109.426	CH(d)	2	0.742	0.742
9	C 9	109.455	CH(d)	2	0.733	0.733
10	C 10	109.637	CH(d)	2	0.985	0.985
11	C 11	118.891	C(s)	1	0.293	0.279
12	C 12	119.438	C(s)	1	0.25	0.207
13	C 13	120.014	C(s)	1	0.292	0.329
14	C 14	120.261	C(s)	1	0.296	0.254
15	C 15	138.981	C(s)	1	0.472	0.472
16	C 16	138.988	C(s)	1	0.472	0.472
17	C 17	139.214	C(s)	1	0.261	0.218
18	C 18	139.695	C(s)	1	0.211	0.156
19	C 19	145.349	C(s)	2	0.94	0.94
20	C 20	145.357	C(s)	2	0.94	0.94
21	C 21	145.524	C(s)	2	1	1
22	C 22	145.531	C(s)	2	1	1
23	C 23	165.344	C(s)	1	0.263	0.134
24	C 24	166.211	C(s)	1	0.246	0.162
25	C 25	166.75	C(s)	1	0.235	0.12
26	C 26	167.209	C(s)	1	0.243	0.15

COSY

#	Label	F2(ppm)	F1(ppm)	F2	F1 Labels	Abs.	Distance

				Labels		Volume	
1	1	5.461	6.107	H 4 (5.461)	H 6 (6.107)	23300.0664	2-3 bond(s)
2	6	4.025	4.595	H 2 (4.025)	H 3 (4.595)	21114.998	2-3 bond(s)
3	4	6.107	5.461	H 6 (6.107)	H 4 (5.461)	20127.7793	2-3 bond(s)
4	7	4.595	4.025	H 3 (4.595)	H 2 (4.025)	19188.8398	2-3 bond(s)
5	8	5.601	3.989	H 5 (5.601)	H 1 (3.989)	14146.1895	2-3 bond(s)
6	3	3.989	5.601	H 1 (3.989)	H 5 (5.601)	10961.8721	2-3 bond(s)
7	5	5.601	5.461	H 5 (5.601)	H 4 (5.461)	6749.3555	2-3 bond(s)
8	2	5.461	5.601	H 4 (5.461)	H 5 (5.601)	6530.7437	2-3 bond(s)

HSQC

#	Label	F2(ppm)	F1(ppm)	F2 Labels	F1 Labels	Abs. Volume	Distance
1	9	3.989	68.646	H 1 (3.989)	C 2 (68.672)	981.4712	1 bond(s)
2	6	4.025	75.663	H 2 (4.025)	C 5 (75.645)	1122.0659	1 bond(s)
3	10	4.595	63.024	H 3 (4.595)	C 1 (63.017)	556.5446	1 bond(s)
4	8	5.461	71.39	H 4 (5.461)	C 3 (71.404)	601.6603	1 bond(s)
5	7	5.601	75.487	H 5 (5.601)	C 4 (75.499)	865.1307	1 bond(s)
6	5	6.107	92.922	H 6 (6.107)	C 6 (92.936)	1736.0867	1 bond(s)
7	1	6.931	109.626	H 7 (6.931)	C 10 (109.637)	8504.6973	1 bond(s)
8	2	7.027	109.451	H 8 (7.027)	C 9 (109.455)	4289.5918	1 bond(s)
9	3	7.04	109.412	H 9 (7.040)	C 8 (109.426)	4999.0654	1 bond(s)
10	4	7.123	109.265	H 10 (7.123)	C 7 (109.273)	6294.687	1 bond(s)

HMBC

#	Label	F2(ppm)	F1(ppm)	F2 Labels	F1 Labels	Abs. Volume	Distance
1	15	7.123	138.947	H 10 (7.123)	C 15 (138.981), C 16 (138.988)	2578.168	2-3 bond(s)
2	10	7.04	145.53	H 9 (7.040)	C 21 (145.524), C 22 (145.531)	2507.2991	2-3 bond(s)
3	14	6.931	139.192	H 7 (6.931)	C 17 (139.214)	2404.2615	2-3 bond(s)
4	16	7.04	138.947	H 9 (7.040)	C 15 (138.981), C 16 (138.988)	2239.0151	2-3 bond(s)
5	3	7.04	166.705	H 9 (7.040)	C 25 (166.750)	1924.3335	2-3 bond(s)
6	13	7.027	139.635	H 8 (7.027)	C 18 (139.695)	1840.6887	2-3 bond(s)
7	7	7.027	165.329	H 8 (7.027)	C 23 (165.344)	1664.7081	2-3 bond(s)
8	1	7.123	167.147	H 10 (7.123)	C 26 (167.209)	1429.6616	2-3 bond(s)
9	5	6.931	166.164	H 7 (6.931)	C 24 (166.211)	1407.8229	2-3 bond(s)
10	11	7.027	145.284	H 8 (7.027)	C 19 (145.349), C 20 (145.357)	1291.1885	2-3 bond(s)
11	9	7.123	145.53	H 10 (7.123)	C 21 (145.524), C 22 (145.531)	881.2808	2-3 bond(s)
12	12	6.931	145.284	H 7 (6.931)	C 19 (145.349), C 20 (145.357)	773.814	2-3 bond(s)
13	8	6.107	165.329	H 6 (6.107)	C 23 (165.344)	706.5392	2-3 bond(s)
14	24	5.601	71.39	H 5 (5.601)	C 3 (71.404)	599.7969	2-3 bond(s)
15	23	5.461	75.487	H 4	C 4	584.8479	2-3

				(5.461) H 1	(75.499) C 5		bond(s) 2-3
16	22	3.989	75.663	(3.989) H 9	(75.645) C 13	581.7849	bond(s) 2-3
17	18	7.04	119.912	(7.040) H 4	(120.014) C 6	506.7276	bond(s) 2-3
18	21	5.461	92.922	(5.461) H 8	(92.936) C 11	405.4531	bond(s) 2-3
19	20	7.027	118.831	(7.027) H 10	(118.891) C 14	352.0018	bond(s) 2-3
20	17	7.123	120.207	(7.123) H 5	(120.261) C 2	300.1852	bond(s) 2-3
21	25	5.601	68.646	(5.601) H 7	(68.672) C 12	294.428	bond(s) 2-3
22	19	6.931	119.372	(6.931) H 5	(119.438) C 25	281.6629	bond(s) 2-3
23	4	5.601	166.705	(5.601) H 4	(166.750) C 24	219.5252	bond(s) 2-3
24	6	5.461	166.164	(5.461) H 3	(166.211) C 2	189.325	bond(s) 2-3
25	26	4.595	68.646	(4.595) H 3	(68.672) C 26	143.0211	bond(s) 2-3
26	2	4.595	167.147	(4.595)	(167.209)	119.5871	bond(s)

Personnel Involved

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TECHNICAL REPORT – PART III

Anticancer and Anti-Inflammatory Potential of the Bearberry-Leaf *(Arctostaphylos uva-ursi L. Sprengel)* Extract, its Low Molecular-Weight Phenolics Fraction and Tannin Fraction

Executive Summary

The crude bearberry-leaf (*Arctostaphylos uva-ursi* L. Sprengel) extract (BBE) was fractionated into a low molecular-weight phenolic fraction, also referred to as a bearberry-leaf non-tannin (BBNT) fraction, and a bearberry-leaf tannin (BBT) fraction as described in Part I of this report. The present work was undertaken to investigate the anticancer and anti-inflammatory potential of three products BBE, BBT and BBNT *in vitro* using cancer cell lines. For anticancer studies, human breast, prostate, colon and skin cancer cell models were employed. Data indicated that BBT inhibited the proliferation of MC-7 breast cancer (estrogen receptor positive), MDA-MBA-435 breast cancer (estrogen receptor negative), DU-145 prostate cancer (androgen receptor negative), HT-29 colon cancer and SK-MEL-5 skin cancer cells best with 50% inhibitory concentrations (IC_{50}) of 4.0, 6.0, 4.7, 1.5 and 2.3 µg/mL, respectively (Table 1). For most cancer cells tested, BBE performed almost as well as the BBT fraction, unlike the BBNT fraction, which was generally weakest. For the anti-inflammatory studies, the objective was to investigate the cytokine production and the ability of the three compounds, BBE, BBT and BBNT, to inhibit cytokine production by various cancer cell lines *in vitro*. It was observed that BBE and BBT were equally effective in inhibiting the cytokine secretion by various cancer cell lines. The only differences between the two compounds were BBE was more effective in inhibiting the IL-12 secretion by MCF-7 cells and BBT was more effective in inhibiting the IL-1β and IL-8 secretion by SkMel-5 cells. Although BBNT showed some inhibition of cytokine secretion by various cells, it was overall least effective. BBE, BBT and BBNT also selectively up regulated TGF-β and ICAM-1 in addition to other cytokines at different concentrations and time points suggesting that they may be beneficial in reducing the tumor size and metastasis if used at an optimum concentration. The results are quite promising; moreover, further experiments should be done to investigate their effect on cytokine secretion and tumor growth *in vivo*.

Background

The role of infectious agents has been described in various cancers. In cases of human leukemia, *Mycoplasma*, *Salmonella*, *Mycobacteriu,* pseudorabies virus and adenovirus antigens have been detected in the patient's sera (*1*). Hepatitis B and C cause hepatic necrosis followed by hepatocellular, B cell and gastric malignancies (*2-5*). It has been confirmed by the fact that antiviral therapy of hepatitis C infection led to the regression of virus-associated B cell lymphoma (*6*). Adenoviral infection has been associated with childhood leukemia (*7*) and cytomegalovirus infection with testicular cancer (*8*). *Helicobacter pylori* infection is associated with gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma and eradication of the infection has been observed to reverse the neutropenia (*9,10*).

In response to pathogens, a protective inflammatory response is initiated by the host. Cells of the immune system migrate to the area of infection and produce inflammatory cytokines. Initially, cells of the innate immune system (*i.e.*, macrophages, neutrophils, NK cells) become involved, followed by the activation of cells of the adaptive immune system. These include antigen-presenting cells (APCs), T and B cells, which play an important role in propagating the inflammatory response. T cells play a major role in antitumor immune responses. Key regulators of T cell-mediated response are the T helper (Th) cells that secrete the cytokines orchestrating this response. The two subtypes Th1 and Th2 cells produce cytokines stimulating cellular and humoral immune responses. Th2 cells secrete IL-10, IL-4, IL-5 and IL-13. These cytokines are antagonistic to Th1 type cytokines (TNF-α, IL-2, IFN-γ, IL-6 and IL-12). Excessive production of either type of cytokine results in homeostatic imbalance necessary to maintain a balance of cellular and humoral immune responses.

Besides regulating innate and adaptive immune responses, cytokines are involved in cell growth and differentiation. Normally, the secretion of cytokines is local and is for limited duration for regulating self or adjacent cell functions. Although the activity of cytokines is strongly regulated, there is evidence that cytokines contribute to inflammatory autoimmune diseases and malignancies (*11-14*).

Similar to activated T cells, various tumor cells secrete cytokines like IL-10, IL-6, IL-8, IL-13 and TGF-β serving as autocrine and/or paracrine growth factors for the cancer (*15-29*). This secretion is often constitutive resulting in high serum levels of cytokines in malignancies including melanoma, non-small cell lung carcinoma, renal cell carcinoma and bladder carcinoma (*15-30*). Secretion of IL-10, the most potent Th2 polarizing cytokine, by the tumor cells in the tumor environment (*21*) suppresses the tumoricidal activity of macrophages (*31*), blocks presentation of tumor antigens to professional APCs (*32-34*), and inhibits tumor-specific cytotoxic T cells (*35*).

Since inflammation and inflammatory cytokines play an important role in the progression of cancer inhibition of cytokine secretion by tumor cells may result in the reduction in tumor size and slower progression of cancer. The present study was undertaken to investigate the effect of three products on the secretion of inflammatory markers like cytokines, chemokines and adhesion molecules using six cancer cell lines.

Materials and Methods

Experimental Protocol: Cells were grown to confluency and then collected. Anticancer studies were performed according to Guthrie *et al.* (*36*). Cells were suspended in relevant media and the cell count was adjusted to $1x10^4$ cells/mL. Cells (1mL/well; $1x10^4$) were plated in 24 well flat bottom plates and incubated for 24, 48 and 72 h. Extracts (*i.e.*, BBE, BBT and BBNT) were added to the cultures at 10, 25 and 50 µg/ml for BBE and BBNT and 2, 10, 20 µg/ml for BBT to get the final concentrations of 5, 12.5 and 25 µg/ml for BBE and BBNT and 1, 5 and 10 µg/ml

for BBT. Plates were incubated at 37°C and 5% CO_2. Supernatant were collected after desired culture time and assayed for the presence of following inflammatory markers: Cytokines: (a) TNF-α; (b) IFN-g; (c) IL-1β; (d) IL-6; (e) TGF- β; (f) IL-8; & (g) IL-12; Chemokines: (a) MIP-1α; & (b) RANTES; Adhesion Molecules: (a) ICAM-1; and C-Reactive Protein (CRP).

Results

Inhibition (IC_{50} values) of various human cancer cells with the bearberry-leaf extract (BBE), bearberry-leaf non-tannin (BBNT) fraction and bearberry-leaf tannin (BBT).

Sample	MCF-7[1]	MDA[2]	DU-145[3]	HT-29[4]	SK-MEL-5[5]
BBE	12.9	14.1	13.2	4.9	3.7
BBNT	6.0	20.5	15.2	13.6	4.2
BBT	4.0	6.0	4.7	1.5	2.3

[1] MCF-7, breast cancer (estrogen receptor positive); [2]MDA-MBA-435, breast cancer (estrogen receptor negative); [3]DU-145, prostate cancer (androgen receptor negative); [4]HT-29, colon cancer; [5]SK-MEL-5, skin cancer cells.
For IC_{50} (μg/mL) values a lower value indicates stronger anticancer activity.

Effect of BBE, BBT and BBNT on TNF-α secretion by various cancer cell lines: It was observed that breast cancer cell line (MCF-7) secreted small amounts of TNF-α after 24 h of incubation and the amounts decreased gradually up to 72 h. Addition of BBE and BBNT to the cultures down regulated the secretion of TNF-α significantly after 24 h ($p<0.027$). The effect of BBE was not observed after 72 h of culture. BBNT slightly up regulated the secretion of TNF-α but by 72 h the highest concentration inhibited the secretion again but it was not statistically significant. BBT, on the other hand, did not have a significant effect on the secretion of TNF-α (Figure 1).

Another breast cancer cell line MDA-MB 435 secreted larger amounts of TNF-α after 24h in culture and the amounts decreased with increasing culture time. It was observed that addition of BBE, BBT and BBNT almost completely inhibited the secretion of TNF-α by these cells after 24 h of culture ($p<0.014$). There was a significant increase in TNF-α secretion after 48 h with all three compounds ($p<0.047$) but the levels of TNF-α were comparatively very low. A significant inhibition with BBE was observed after 72 h at the two lower concentrations ($p<0.019$) but the highest concentration of BBE, BBT and BBNT did not have any effect (Figure 2).

Effect of the compounds was tested on TNF-α secretion by prostate cancer cell line (DU145). This cell line secreted very low amounts of TNF-α and no significant effect was observed after addition of the compounds for 24 h. BBE, BBT and BBNT significantly increased the secretion of TNF-α after 48h in a concentration dependent manner ($p<0.044$), but the levels were very low. No significant differences were observed after 72 h of culture (Figure 3).

A colon cancer cell line (HT-29) was also tested for the effect of the compounds. HT-29 cell secreted small amounts of TNF-α and almost no TNF-α was detected after 72 h of culture. Addition of BBT down regulated the secretion of TNF-α after 24 h of culture at the higher concentrations ($p<0.044$). The effect of BBT was observed with the highest concentration after 48 h ($p=0.002$), but there was a significant increase ($p<0.001$) in TNF-α secretion after 72 h of culture. BBE at the higher concentrations inhibited the secretion of TNF-α significantly after 48 h of culture ($p<0.033$) but the effects after 24 and 72 h were not significant. BBNT significantly inhibited secretion of TNF-α at the highest concentration after 48 h ($p<0.035$) but up regulated the secretion of TNF-α after 72 h ($p<0.022$) compared to the control (Figure 4).

Effect of compounds was also tested on skin melanoma cell line (SkMel-5). These cells secreted small amounts of TNF-α after 24 and 48 h of culture. After 72 h of culture the secretion of TNF-α was almost negligible. Addition of BBE and BBT to the culture inhibited the secretion of TNF-α in a concentration dependent manner after 24 and 72 h culture, but the effect was significant only for BBE at 72 h ($p<0.042$). No effect was observed after 48 h of culture. BBNT did not have significant inhibitory effect at any time point (Figure 5).

Effect of BBE, BBT and BBNT on IL-1β secretion by various cancer cell lines: Addition of BBE, BBT and BBNT up regulated the secretion of IL-1β by MCF-7 cells after 24 h of culture but the increase was significant for BBE and BBT only ($p<0.015$). However, BBE (higher concentrations) and BBT significantly inhibited the secretion after 48 h of culture ($p<0.032$) while BBNT did not have any significant effect. After 72 h of culture BBE (two lower concentrations) and BBT (lowest concentration) still showed significant inhibition ($p<0.012$) inhibition and in addition BBNT showed a significant inhibition ($p=0.006$) of IL-1β secretion at the highest concentration (Figure 6).

Compound BBT almost completely inhibited the secretion of IL-1β by MDA-MB 435 cells after 24 h of culture while BBE and BBNT did not have any effect. The inhibitory effect of BBT was not observed at subsequent time points. BBNT significantly up regulated the secretion of IL-1β after 48 h of culture ($p<0.039$). On the other hand, BBE showed some inhibition after 48 h of culture but the levels were very low. There was a significant increase in IL-1β secretion in response to BBE at the highest concentration after 72 h ($p=0.002$). No significant effects were observed after 72 h for BBT and BBNT (Figure 7).

The prostate cancer cell line (DU145) was incubated with BBE, BBT or BBNT and IL-1β secretion was measured. Small amount of IL-1β was secreted by these cells and amount decreased with increasing time. Addition of BBE, BBT or BBNT did not have any significant effect after 24 h, and the secretion of IL-1β was significantly up regulated after 48 h of culture in response to three compounds ($p<0.032$). However, after 72 h, BBT significantly inhibited the IL-1β secretion by DU145 cells at all the concentrations ($p<0.009$). The highest concentration of

BBNT significantly inhibited the secretion of IL-1b after 72 h of culture (p=0.040). The highest concentration of BBE was also inhibitory but the effect was not statistically significant (Figure 8).

Incubation of colon cancer cell line (HT-29) with BBT (highest concentration) and BBNT (all the concentrations) significantly inhibited the secretion of IL-1β (p<0.001) while BBE did not have any effect after 24 h of culture. Similar trend was observed after 48 and 72 h culture for BBT and BBNT (p<0.011). On the other hand, BBE significantly up regulated the secretion of IL-1β (p=0.022) after 48 h of culture at the highest concentration (Figure 9).

Addition of BBE, BBT and BBNT to skin melanoma cells (SkMel-5) inhibited the secretion of IL-1β significantly after 72 h of culture (p<0.019). A decrease in IL-1β was observed after 24 h for all three compounds (p<0.004) with BBNT being most effective (Figure 10).

Effect of BBE, BBT and BBNT on IFN-γ secretion by various cancer cell lines: Various cancer cell lines were incubated with BBE, BBT and BBNT for 24, 48 and 72 h time points. Culture supernatants were collected and assayed for the presence of IFN-γ. A decrease in IFN-γ secretion by MCF-7 cells was observed after 24 and 48 h of culture with all three compounds, but the effect was significant only after 48 h (p<0.05). However, the secretion was up regulated after 72 h of culture but the highest concentrations of BBNT still showed some inhibition (p=0.004; Figure 11).

Secretion of IFN-γ by MDA-MB 435 cells was not affected by addition of BBE and BBNT for 24 h but BBT showed an increase in secretion. Incubation for 48 and 72 h up regulated the secretion of IFN-γ in a concentration dependent manner for all three compounds but the increase was only significant for BBT (p<0.029; Figure 12).

DU145 cells secreted IFN-γ after 24 h in culture and the levels went down after 48 and 72 h cultures. Addition of BBE, BBT and BBNT significantly inhibited the secretion of IFN-γ by DU145 cells after 24 h of culture (p<0.001). Only BBE showed a significant decrease in IFN-γ secretion after 48 h at the highest concentration (p=0.045) while BBNT significantly up regulated the secretion (p<0.015). No significant effects were observed after 72 h (Figure 13).

The colon cancer cell line HT-29 secreted small amounts of IFN-γ and the secretion was up regulated significantly by BBE (two higher concentrations) and BBNT after 24 h of culture (p<0.025). BBNT inhibited the secretion of IFN-γ significantly at 48 h (p<0.005) but not at 72 h. BBE up regulated the secretion of IFN-γ by 48 h although not significantly but significantly inhibited again at 72 h at higher concentrations (p<0.002). BBT did not have any significant effect after 48 h but showed significant inhibition after 72 h in culture (p<0.0001; Figure 14).

There was no effect by the addition of BBT or BBNT on IFN-γ secretion by SkMel-5 cells after 24 h of culture but BBE up regulated the secretion significantly at the highest concentration (p=0.016). Both BBE and BBNT up regulated the secretion significantly after 48 h (p<0.048). All the compounds inhibited secretion of IFN-γ by SkMel-5 cells at 72 h but BBT was found to be most effective (p<0.004; Figure 15).

Effect of BBE, BBT and BBNT on IL-8 secretion by various cancer cell lines: BBE, BBT and BBNT inhibited the secretion of IL-8 by MCF-7 cells after 24 h of culture; however, inhibition with only BBE was statistically significant (p<0.049). BBE was found to be most effective and BBT only inhibited the secretion at the highest concentration. No significant effects were observed at subsequent time points with slight increases in the secretion of IL-8 but the levels were negligible (Figure 16).

The breast cancer cell line MDA-MB 435 secreted comparatively larger amounts of IL-8 and the levels increased with time. Addition of BBE and BBT inhibited the secretion of IL-8 in a concentration dependent manner after 48 h (p<0.024) but BBNT did not have any effect. After 72 h of culture all the compounds inhibited the secretion of IL-8 significantly (p<0.034) except the lowest concentration of BBE (p=0.482). No effects were observed at 24 h suggesting that a longer exposure of these cells to BBE, BBT and BBNT is required for inhibiting the secretion of IL-8. (Figure 17).

The prostate cancer cell line (DU145) also secreted IL-8 and the levels increased with time. Addition of BBE at the two lower concentrations significantly inhibited the IL-8 secretion (p<0.024) but BBT and BBNT did not have any effect after 24 h. After 48 and 72 h of culture no significant effect were observed with BBE and BBT but BBNT significantly inhibited the secretion (p<0.015; Figure 18).

Addition of BBE, BBT and BBNT did not have any significant effect on the secretion of IL-8 by HT-29 cells after 24 h of culture. All the compounds inhibited the secretion after 48 h of culture (p<0.05). After 72 h of culture the highest concentrations of BBE and all the concentrations of BBT inhibited the IL-8 secretion significantly (p<0.028), but BBNT increased the secretion of IL-8 (p<0.018; Figure 19).

The skin melanoma cell line (SkMel-5) secreted IL-8 and the secretion levels increased with increasing culture time. Addition of BBT to the culture inhibited the secretion of IL-8 at all time points in a concentration dependent manner (p<0.008) while BBE showed a concentration dependent inhibition after 24 (two higher concentrations) and 72 h (all the concentrations) of culture (p<0.014). BBNT inhibited the secretion of IL-8 after 24 h (at all concentrations), 48 and 72 h (two lower concentrations) of culture (p<0.021) but increased the secretion after 48 and 72 h (at the highest concentration) of culture. The increase after 72 h with BBNT was significant (p<0.0001; Figure 20).

Effect of BBE, BBT and BBNT on IL-12 secretion by various cancer cell lines: Further the effect of BBE, BBT and BBNT on IL-12 secretion was investigated. It was observed that BBE and BBT inhibited the secretion of IL-12 at the highest concentration after 24 h of culture ($p<0.043$). Similar results were observed for BBE ($p=0.041$) after 48 h but BBT did not inhibit the secretion significantly. After 72 h of culture, both BBE and BBT inhibited the secretion significantly at the two higher concentrations ($p<0.009$). BBNT did not have any significant effect on the secretion of IL-12 after 24 and 48 h, but after 72 h the highest concentration significantly up regulated the secretion ($p=0.019$; Figure 21).

BBT significantly inhibited the secretion of IL-12 by MDA-MB-235 cells after 24 h of culture at the two higher concentrations ($p<0.014$). After 48 and 72 h of culture, the inhibition was observed only with the highest concentration of BBT ($p<0.05$). BBE and BBNT did not have any significant effect on IL-12 secretion by MDA-MB-235 cells except slight inhibition observed at 48 and 72 h ($p>0.156$; Figure 22).

The prostate cancer cell line (DU145) secreted small amounts of IL-12 with a slight increase after 72 h of culture. There was a concentration dependent decrease in IL-12 secretion at 24 h after addition of BBE and BBT to the culture ($p<0.05$). However, all the compounds increased the secretion of IL-12 by DU145 cells after 48 h ($p<0.002$). Significant decreases were observed at 72 h with BBE and BBT ($p<0.041$) while BBNT did not have any significant effect (Figure 23).

Addition of BBE (two higher concentrations), BBT and BBNT (all the concentrations) significantly inhibited the secretion of IL-12 ($p<0.035$) after 24 h by HT-29 cells. After 48 and 72 h significant inhibition ($p<0.037$) was observed only with BBE (the highest concentration) and BBT (two higher concentrations). BBNT did not have any significant effect after 48 h but the two higher concentrations of BBNT inhibited the secretion of IL-12 after 72 h of culture ($p<0.023$; Figure 24).

SkMel-5 cells secreted very small amounts of IL-12. Addition of BBE (two higher concentrations) and BBT at the highest concentration inhibited the secretion at 24 h ($p<0.027$). After 48 h, both BBE and BBT inhibited the secretion of IL-12 at two higher concentrations ($p<0.038$). BBE significantly inhibited the secretion at all the concentrations after 72 h ($p<0.039$) while BBT did not have any effect. BBNT did not have any significant effect on the secretion of IL-12 by SkMel-5 cells (Figure 25).

Effect of BBE, BBT and BBNT on TGF-β secretion by various cancer cell lines: The MCF-7 cell line secreted small amounts of TGF-β which increased with increasing culture time. BBE inhibited the secretion of TGF-β at the highest concentration ($p=0.027$) after 72 h in culture but up regulated ($p<0.040$) the secretion at the other time points. BBT inhibited the TGF-β secretion at the highest concentration after 24 and 48 h ($p<0.017$). BBNT significantly up regulated

($p<0.05$) the secretion after 24 h (two higher concentrations) and 48 h (the highest concentration) but the up regulation was not significant ($p>0.276$) after 72 h (Figure 26).

MDA-MB 435 cells did not secrete detectable levels of TGF-β at 24 and 48 h of culture. Significant amounts of TGF-β were detected after 72 h in culture and secretion was inhibited by BBE and BBT completely ($p<0.005$). BBNT inhibited the secretion slightly at the highest concentration but the decrease was not statistically significant (Figure 27).

The prostate cancer cell line (DU145) did not secrete detectable amounts of TGF-β after 24 h but culture with BBNT at the highest concentration up regulated the secretion ($p=0.026$) while BBE and BBT did not have any effect. Small amount of TGF-β was secreted after 48 h of culture and the lowest concentrations of BBE and BBT up regulated the secretion ($p<0.039$) while the highest concentration of BBNT up regulated the secretion ($p=0.006$). After 72 h, the higher two concentrations of BBE and BBT inhibited the secretion significantly ($p<0.05$) while the highest concentration of BBT increased the secretion ($p=0.002$). BBNT did not have any significant effect after 72 h of culture (Figure 28).

Colon cancer cells (HT-29) did not secrete any detectable TGF-β after 24 and 72 h of culture; however, TGF-β was detected after 48 h of culture. BBE inhibited the secretion after 48 h of culture significantly at all the concentrations ($p<0.018$) while BBT inhibited the secretion at two higher concentrations ($p<0.016$). BBNT did not have any significant effect (Figure 29).

The skin melanoma cell line did not secrete any TGF-β. A significant increase in TGF-β secretion was observed after 24 h with BBT at the lowest concentration ($p=0.031$). After 72 h, BBE (two lower concentrations) and BBNT significantly up regulated the secretion ($p<0.015$; Figure 30).

Effect of BBE, BBT and BBNT on ICAM-1 secretion by various cancer cell lines: The breast cancer cell line MCF-7 secreted low levels of ICAM-1 which went down with increasing culture time. Addition of the three compounds inhibited the secretion of ICAM-1 at the higher concentrations after 24 h but the decrease was not significant ($p>0.120$). No significant effects were observed after 48 h of culture. After 72 h of culture, the lowest concentration of BBT and the highest concentration of BBNT up regulated ICAM-1 ($p<0.005$) while BBE did not have any significant effect (Figure 31).

Similar amounts of ICAM-1 were secreted by MDA-MB-235 cells; however, no significant differences were observed with any of the compounds after 24 and 48 h ($p>0.109$). After 72 h, BBE (all the concentrations) and BBT (two lower concentrations) up regulated the ICAM-1 significantly ($p<0.009$). BBNT did not have any significant effect (Figure 32).

The prostate cancer cell line (DU145) secreted comparatively larger amounts of ICAM-1. Addition of BBE at the lowest concentration and BBNT at two higher concentrations inhibited

the secretion (p<0.025) while BBT did not have any significant effect. After 48 h, BBE (two higher concentrations), BBT and BBNT (all the concentrations) significantly up regulated the secretion of ICAM-1 (p<0.049). BBE and BBT up regulated the secretion at two higher concentrations after 72 h while BBNT up regulated ICAM-1 at the highest concentration (p<0.049; Figure 33).

BBNT inhibited the secretion of ICAM-1 by HT-29 cells after 24 h (p<0.014) but BBE and BBT did not have any effect. After 48 h, BBE (two higher concentrations), BBT (all the concentrations) and BBNT (the lowest and the highest concentration) inhibited the ICAM-1 secretion significantly (p<0.005). However, after 72 h only BBT at two higher concentrations was able to inhibit the secretion significantly (p<0.004; Figure 34).

BBE inhibited the secretion of ICAM-1 by SkMel-5 cells after 24 h at two higher concentrations (p<0.012) but there was a significant increase in ICAM-1 after 48 and 72 h with BBE (p<0.018). BBT did not have any significant effect after 24 h but a significant increase was observed after 48 (two higher concentrations) and 72 h (all the concentrations) with BBT (p<0.015). BBNT did not have any significant effect after 24 and 72 h of culture but the highest concentration up regulated the secretion of ICAM-1 after 48 h (p=0.024; Figure 35).

Effect of BBE, BBT and BBNT on MIP 1-α secretion by various cancer cell lines: MIP-1-α was not secreted by any of the cell lines tested in this study.

Effect of BBE, BBT and BBNT on RANTES secretion by various cancer cell lines: RANTES was not secreted by any of the cell lines tested in this study.

Effect of BBE, BBT and BBNT on CRP secretion by various cancer cell lines: No detectable amounts of CRP were secreted by any of the cancer cell lines tested in this study.

Discussion and Conclusions

Cytokines are a diverse group of intercellular signaling peptides and glycoproteins that regulate local and systemic inflammatory and immune responses (*37*). Unlike hormones, cytokines are produced by various immunomodulatory cells, such as subsets of T cells activated macrophages and fixed histiocytes (*38*). Cytokine production follows receptor mediated T cell activation and is short lived. Activated macrophages predominantly produce pro-inflammatory cytokines, including IL-1, TNF-α and IFN-γ. Functionally cytokines are classified as Th1 or pro-inflammatory (IL-1, IL-2, IFN-γ and tumor necrosis factor-a) and Th2 or anti-inflammatory (IL-4, IL-6 and IL-10; *39*). Cytokines may be produced by various cells, such as neutrophils, mast cells, eosinophils, dendritic cells, macrophages, B cells, CD4 and CD8 T cells.

Tumor cells produce various cytokines and chemokines that attract leukocytes. The inflammatory component of a developing neoplasm may include a diverse leukocyte population all of which are capable of producing an array of cytokines such as TNF-α, interleukins and interferons (IFNs; *40, 41*). Some tumor cells not only regulate their chemokine expression to help recruit inflammatory cells, but also use these factors to further the tumor growth and progression. Melanoma is perhaps the best example in which chemokines (e.g. GROα/CXCL1, GROβ/CXCL2, GROγ/CXCL3 and IL-8/CXCL8) have been shown to exert autocrine control over neoplastic cell proliferation (*42*).

There has been less research on the role of cytokines and other inflammatory mediators in tumor development. Mantovani *et al.* demonstrated that serum levels of pro-inflammatory cytokines IL-2, IL-6, TNF-α, leptin and C-reactive protein were higher in 82 advanced cancer patients than in 36 controls (*43*). The concentration of C-reactive protein in plasma was reported to correlate inversely with survival in cancer patients with multiple myeloma, melanoma, lymphoma and tumors of the ovary, pancreas and gastro-intestinal tract (*44*).

The present study was undertaken to investigate the cytokine production and the ability of BBE, BBT and BBNT to inhibit the cytokine production by various cancer cell lines *in vitro*. It was observed that BBE and BBT were equally effective in inhibiting the cytokine secretion by various cancer cell lines. The only differences between the two compounds were BBE was more effective in inhibiting the IL-12 secretion by MCF-7 cells and BBT was more effective in inhibiting the IL-1β and IL-8 secretion by SkMel-5 cells. Although BBNT showed some inhibition of cytokine secretion by various cells, it was overall least effective.

TGF-beta is a multi-functional cytokine. At a cellular level, it mediates cellular proliferation, growth arrest, differentiation and apoptosis. TGF-β regulates normal embryonic development, angiogenesis in tumor tissues, malignant transformation and immune surveillance. TGF-*β* can play two different and opposite roles with respect to the process of malignant progression. During early stages of carcinogenesis, TGF-*β* acts predominantly as a potent tumor suppressor and may mediate the actions of chemopreventive agents such as retinoids and nonsteroidal anti-estrogens. At some point during the development and progression of malignant neoplasms, however, bioactive TGF-*β* may help the tumor cells escape from TGF-*β*-dependent growth arrest. Most of the tumor cells tested in this study did not secrete significant amounts of TGF-β suggesting that its production is down regulated in cancer cells to promote their growth. BBE, BBT and BBNT at different concentrations and time points did up regulate the secretion of TGF-β by these cells suggesting that with an optimum concentration and exposure time these cells can be induced to secrete TGF-β which may in turn down regulate their growth.

ICAM-1 expression has been found to be inversely related to tumor size, lymph node metastasis, tumor infiltration, nuclear pleomorphism, and nuclear grade. Patients with ICAM-1-positive tumors had better relapse-free and overall survival than those with negative tumors (*45*). The

compounds BBE, BBT and BBNT selectively up regulated ICAM-1 at different concentrations and time points suggesting that they may be beneficial in reducing the tumor size and metastasis.

Pro-inflammatory cytokines secreted by tumor cells and other cells of the immune system play an important role in the progression of tumors. The cytokines secreted by tumor cells also modulate the immune system to suppress tumor specific responses. Selective inhibition/up regulation of cytokine production by tumor cells using BBE and BBT may provide a means for suppressing tumor growth and enhancing the anti-tumor responses. However, these compounds also up regulate some cytokines. Therefore, further experiments are required to investigate their effect on cytokine secretion and tumor growth *in vivo*.

Abbreviations

TNF-α	Tumor Necrosis Factor - alpha
IFN-γ	Interferon - gamma
IL-1β	Interleukin 1-beta
IL-6	Interleukin - 6
TGF- β	Transforming Growth Factor - beta
IL-8	Interleukin - 8
IL-12	Interleukin - 12
ICAM-1	Intracellular Adhesion Molecule - 1
MIP-1α	Macrophage Inflammatory Protein-1 alpha
RANTES	Regulated upon Activation, Normal T Expressed and Secreted
APC	Antigen Presenting Cells
Th	T helper cells
CRP	C-Reactive Protein

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Figure 1: Effect of BBE, BBT and BBNT on TNF-α secretion by breast cancer cells (MCF-7).

Figure 2: Effect of BBE, BBT and BBNT on TNF-α secretion by breast cancer cells (MDA-MB-435).

Figure 3: Effect of BBE, BBT and BBNT on TNF-α secretion by Prostate cancer cells (DU-145).

Figure 4: Effect of BBE, BBT and BBNT on TNF-α secretion by colon cancer cells (HT-29).

Figure 5: Effect of BBE, BBT and BBNT on TNF-α secretion by skin melanoma cells (SkMel-5).

Figure 6: Effect of BBE, BBT and BBNT on IL-1β secretion by breast cancer cells (MCF-7).

Figure 7: Effect of BBE, BBT and BBNT on IL-1β secretion by breast cancer cells (MDA-MB-435).

Figure 8: Effect of BBE, BBT and BBNT on IL-1β secretion by Prostate cancer cells (DU-145).

Figure 9: Effect of BBE, BBT and BBNT on IL-1β secretion by colon cancer cells (HT-29).

Figure 10: Effect of BBE, BBT and BBNT on IL-1β secretion by skin melanoma cells (SkMel-5).

Figure 11: Effect of BBE, BBT and BBNT on IFN-γ secretion by breast cancer cells (MCF-7).

Figure 12: Effect of BBE, BBT and BBNT on IFN-γ secretion by breast cancer cells (MDA-MB-435).

Figure 13: Effect of BBE, BBT and BBNT on IFN-γ secretion by Prostate cancer cells (DU-145).

Figure 14: Effect of BBE, BBT and BBNT on IFN-γ secretion by colon cancer cells (HT-29).

Figure 15: Effect of BBE, BBT and BBNT on IFN-γ secretion by skin melanoma cells (SkMel-5).

Figure 16: Effect of BBE, BBT and BBNT on IL-8 secretion by breast cancer cells (MCF-7).

Figure 17: Effect of BBE, BBT and BBNT on IL-8 secretion by breast cancer cells (MDA-MB-435).

Figure 18: Effect of BBE, BBT and BBNT on IL-8 secretion by prostate cancer cells (DU-145).

Figure 19: Effect of BBE, BBT and BBNT on IL-8 secretion by colon cancer cells (HT-29).

Figure 20: Effect of BBE, BBT and BBNT on IL-8 secretion by skin melanoma cells (SkMel-5).

Figure 21: Effect of BBE, BBT and BBNT on IL-12 secretion by breast cancer cells (MCF-7).

Figure 22: Effect of BBE, BBT and BBNT on IL-12 secretion by breast cancer cells (MDA-MB-435).

Figure 23: Effect of BBE, BBT and BBNT on IL-12 secretion by prostate cancer cells (DU-145).

Figure 24: Effect of BBE, BBT and BBNT on IL-12 secretion by colon cancer cells (HT-29).

Figure 25: Effect of BBE, BBT and BBNT on IL-12 secretion by skin melanoma cells (SkMel-5).

Figure 26: Effect of BBE, BBT and BBNT on TGF-β secretion by breast cancer cells (MCF-7).

Figure 27: Effect of BBE, BBT and BBNT on TGF-β secretion by breast cancer cells (MDA-MB-435).

Figure 28: Effect of BBE, BBT and BBNT on TGF-β secretion by prostate cancer cells (DU-145).

Figure 29: Effect of BBE, BBT and BBNT on TGF-β secretion by colon cancer cells (HT-29).

Figure 30: Effect of BBE, BBT and BBNT on TGF-β secretion by skin melanoma cells (SkMel-5).

Figure 31: Effect of BBE, BBT and BBNT on ICAM-1 secretion by breast cancer cells (MCF-7).

Figure 32: Effect of BBE, BBT and BBNT on ICAM-1 secretion by breast cancer cells (MDA-MB-435).

Figure 33: Effect of BBE, BBT and BBNT on ICAM-1 secretion by prostate cancer cells (DU-145).

Figure 34: Effect of BBE, BBT and BBNT on ICAM-1 secretion by colon cancer cells (HT-29).

Figure 35: Effect of BBE, BBT and BBNT on ICAM-1 secretion by skin melanoma cells (SkMel-5).

Personnel Involved

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Summary of Expenses

An official statement of receipts and expenditures obtained from the University of Saskatchewan for project #20030475 for the period April 1, 2003 to February 15, 2006 is attached to this electronic submission.